Topical CpG Enhances the Response of Murine Malignant Melanoma to Dacarbazine

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Malignant melanoma is a potentially fatal skin cancer that is increasing in incidence. Standard chemoimmunotherapy consisting of dacarbazine (DTIC) given with IFN-α has had disappointing results. We describe a chemoimmunotherapy protocol for cutaneous melanoma that combines the administration of DTIC with the topical application of CpG oligodinucleotide (ODN). Subcutaneous B16 melanoma tumors in C57BL/6 mice were treated with intraperitoneal injections of DTIC followed by the topical application of CpG-ODN over the tumors. This therapeutic approach abrogated the growth of established tumors and significantly enhanced survival. Topical CpG application was more effective than intratumoral CpG. Cell depletion studies indicated that the antitumor effect was dependent on both $CD4^+$ and $CD8^+$ cells but not on natural killer (NK) cells. Tumor-specific cytotoxic T-lymphocyte activity was generated in treated animals and was highest in topically treated animals. Immunohistochemical analysis revealed that DTIC, but not CpG, enhanced tumor cell apoptosis. Further, topical CpG induced an expansion of a $B220^+CD8^+$ subset of dendritic cells and a subset of $NK1.1^+$ $CD11c^+$ cells within the tumors. By enhancing both tumor cell death and local immune activation, DTIC/topical CpG chemoimmunotherapy induced an effective T-cell-dependent host–immune response against melanoma.

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INTRODUCTION

The incidence of malignant melanoma is increasing. Dacarbazine (DTIC) is the standard treatment for metastatic disease. Unfortunately, the response rate to this single-agent therapy is limited with little evidence for improved overall survival (Brown and Kirkwood, 2003). As melanoma is an immunogenic tumor, strategies to enhance the immune response to the tumor have been pursued. IFN-α administration is one such strategy. Clinical responses to IFN-α may be more sustained than with standard chemotherapy, but are associated with significant toxicity (Brown and Kirkwood, 2003). Chemoimmunotherapy is a strategy whereby the different and, perhaps, synergistic mechanisms of action of these treatments are combined. Again, such combination treatments have not resulted in increased survival for melanoma patients (Sasse *et al.*, 2007). However, a growing understanding of the mechanisms of immunologic tumor rejection offers hope that more effective therapies can be designed.

Human trials of adoptive T-cell transfer have suggested that T cells and, particularly, $CD8^+$ cytotoxic T lymphocytes (CTL) are important effector cells for melanoma rejection (Dudley and Rosenberg, 2003). Cross-presentation of antigen, where tissue-specific antigen is processed by antigen-presenting cells, is one means of priming $CD8^+$ T cells (Heath and Carbone, 2001). Adjuvants are substances used to enhance immune responses by inducing efficient antigen presentation by dendritic cells (DC). Toll-like receptors (TLR) are a family of 10 microbial pattern recognition receptors that stimulate DC, and TLR agonists have thus been studied extensively as potential adjuvants for use in vaccine design and cancer immunotherapy (Akira *et al.*, 2006). *In vitro* studies have determined that TLR3, -7, -8, and -9 enhance the cross-presentation of antigen and that optimal induction of $CD8^+$ T-cell responses occurs with TLR7, -8, or -9 stimulation (Datta *et al.*, 2003; Lore *et al.*, 2003). Studies comparing the efficacy of TLR7/8 and TLR9 agonists, as vaccine adjuvants in mice, have shown that superior cell-mediated immune responses and antibody responses are obtained with TLR9 adjuvants (Weeratna *et al.*, 2005). As TLR9 is thought to have the narrowest expression profile among TLRs in humans, this may allow a safer adjuvancy profile. For example, when non-human primates are vaccinated with TLR agonists emulsified with protein antigen, local inflammation was detected at the site of injection from 2 to 12 weeks with TLR7/8 agonists but not with TLR9 agonists, despite a similar induction of functional T-cell memory (Wille-Reece *et al.*, 2006). Thus, further exploration of the use of TLR9 agonists in chemo-immunotherapy strategies is warranted.

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Abbreviations: CTL, cytotoxic T lymphocyte; DC, dendritic cell; DTIC, dacarbazine; ODN, oligodinucleotide; PBS, phosphate-buffered saline; TLR, Toll-like receptor; TRP2, tyrosinase-related peptide 2

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Immunostimulatory unmethylated CpG-ODN are potent adjuvants that signal through TLR9 (Hemmi *et al.*, 2000). TLR9 agonists have been administered systemically in animal models and humans to induce antitumor immune responses, but have so far met with limited clinical success and have had associated systemic toxicities such as fever and malaise (Krieg, 2007). We have shown that CpG-ODN may be applied topically to the skin to induce efficient cross-presentation of subcutaneous antigen (Najar and Dutz, 2007). Importantly, this proceeds without the systemic toxicities and cytokine release noted with parenteral administration.

DTIC induces melanoma cell death (Thallinger *et al.*, 2003). Dying tumor cells can be engulfed by antigen-presenting cells leading to the cross-presentation of tumor antigen (Nowak *et al.*, 2003). We thus proposed that topical CpG could induce an efficient immune response to malignant melanoma tumors after DTIC therapy while minimizing systemic toxicity. Here, we show that the application of topical CpG after DTIC chemotherapy allows the effective treatment of established B16 melanoma tumors in mice. This treatment induces CTL against the tumors, and a dramatic therapeutic effect that is both $CD4^+$ and $CD8^+$ cell-dependent. Further, the treatment induces the accumulation of $B220^+CD8^+$ DC within the tumors. This population is similar to type 1 IFN-producing plasmacytoid DCs that are known to release IFN-α locally and to promote cross-presentation of antigens by other DCs (O'Keeffe *et al.*, 2002).

RESULTS

Topical CpG administered in combination with DTIC inhibits melanoma growth and improves survival

B16 is a murine melanoma cell line that is relatively immunoresistant (Seliger *et al.*, 2001). DTIC induces melanoma cell apoptosis within 6 hours of administration and has a short half-life *in vivo* (D'Incan and Souteyrand, 2001). We reasoned that administration of DTIC, 24 hours before TLR9 agonist administration for the therapy of an established tumor, would allow for tumor cell apoptosis and decrease DTIC to levels that do not inhibit T-cell priming. To explore the potential adjuvant role for topical CpG after DTIC therapy of melanoma, C57BL6 mice were inoculated with B16 melanoma tumors. Once a tumor mass was well established, mice were repeatedly treated with DTIC. DTIC is commonly administered to humans intravenously in 3-week cycles to limit hematologic toxicity. DTIC was administered to the mice via intraperitoneal route ($80\,mg\,kg^{-1}$) according to a dosage schedule determined previously (Wack *et al.*, 2001). Intraperitoneal DTIC administration in rodents has pharmacologic effects that are similar to intravenous administration (Paschke *et al.*, 1993).

DTIC therapy was followed by the application of topical CpG (Figure 1) to the skin overlying the tumor (termed epifocal). As topical application of 250 μg of CpG was superior and less toxic than 50 μg subcutaneous CpG for the induction of immune responses to foreign subcutaneous antigen (Najar and Dutz, 2007), we also compared the efficacy of epifocal to intratumoral CpG administration using

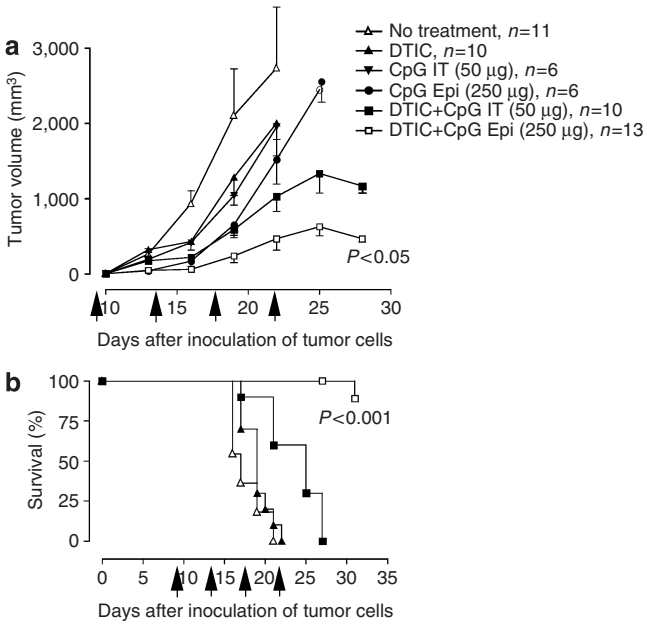

Figure 1. Chemoimmunotherapy with topical or intratumoral CpG and DTIC controls the growth of established B16 melanomas and improves survival. Tumor-bearing mice were treated with dacarbazine (DTIC) followed by CpG application as indicated (either intratumoral—CpG IT or epicutaneously, overlying the tumor—CpG Epi). This cycle was repeated four times at 4-day intervals as indicated by arrows. Mice treated with DTIC and with either epicutaneous ($n=13$) or intratumoral CpG ($n=10$) had less tumor growth than untreated mice ($n=11$) ($P<0.003$ and $P<0.001$, respectively) or mice treated with DTIC alone ($n=10$) (**a**). Survival analysis (**b**): mice were considered terminal when tumors reached $1\,cm^3$ in volume. Using this outcome measure, mice treated with DTIC and with either topical or IT CpG survived significantly longer than untreated mice or mice treated with DTIC alone ($P<0.001$).

similar doses in groups of 10–13 mice. DTIC therapy modestly decreased tumor growth, as did topical or intratumoral administration of CpG. However, sequential administration of DTIC and CpG, either intratumorally or topically, significantly inhibited tumor growth ($P<0.003$ and $P<0.001$, respectively, compared with untreated). Groups of mice treated with intratumoral CpG and DTIC demonstrated a higher tumor volume than topically treated mice (at day 28 from tumor injection mean volume ± SE: 1,050 ± 100 vs $290 \pm 95\,mm^3$, respectively). In a survival analysis, the topical administration of CpG after DTIC therapy clearly outperformed sequential intratumoral administration ($P<0.001$). Thus, topical TLR9 agonist therapy after DTIC induces an effective antitumor response against dermal melanoma.

CpG and DTIC induce a cytotoxic T-cell response against established dermal melanoma tumors

To determine whether CTL activity was generated against the B16 tumors by our chemoimmunotherapy regimen, we tested splenocytes from the treated mice using a standard chromium release assay. Splenocytes were stimulated once *in vitro* by B16 tumor cells and then tested for B16-specific cytotoxicity (Figure 2). Minimal cytotoxicity was noted in untreated animals or animals treated with DTIC alone. Significant

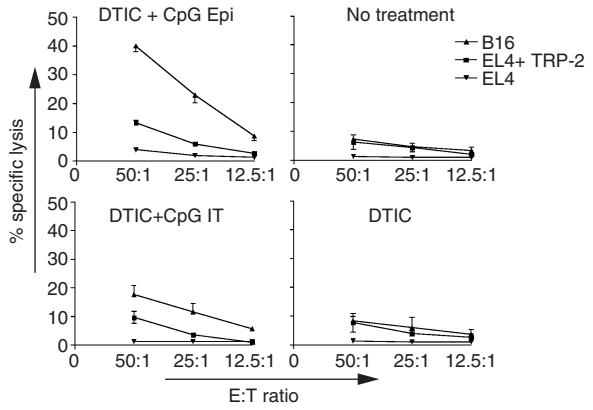

Figure 2. Chemoimmunotherapy using topical or intratumoral CpG and DTIC induces melanoma-specific CTL in mice. Splenocytes from mice with established B16 tumors that were treated with DTIC and with either intratumoral or epicutaneous CpG, or were left untreated, were re-stimulated with irradiated B16 cells once *in vitro*. Chromium release assays were performed against B16 melanoma or EL4 cells with or without TRP2 peptide. Mice treated with DTIC and CpG topically mounted CTL responses to both B16 tumor and TRP2 peptide. The data are expressed as the mean ± SD of triplicate cultures and are representative of three independent experiments.

cytotoxicity was noted against the B16 tumor line but not against the unrelated thymoma cell line EL4 in animals treated with DTIC and sequential CpG. Tyrosinase-related peptide 2 (TRP2) is an immunodominant nonamer melanoma epitope presented by the K^b MHC molecule (Bloom *et al.*, 1997). Mice treated with the combination DTIC and CpG exhibited cytotoxicity to EL4 pulsed with the TRP2 peptide but not EL4 alone, demonstrating the presence of TRP2-specific $CD8^+$ CTL within the splenocyte population. Roughly twofold higher B16 and TRP2-specific cytotoxicity was reproducibly noted in animals treated with topical CpG than those treated with intratumoral CpG (41 vs 18% for B16 target responses). This indicates that chemoimmunotherapy with topical CpG after DTIC induces systemic cellular cytotoxicity against melanoma and primes immunodominant epitope-specific CTL against the tumor. Further, topical TLR9 agonist therapy outperforms intratumoral TLR9 agonist administration in this regard.

DTIC induces tumor apoptosis, whereas topical CpG enhances $B220^+CD8^+$ DC infiltration

TLR9 agonists such as CpG may sensitize tumors to chemotherapy or may directly induce tumor cell death (Wang *et al.*, 2006). To evaluate the effect of DTIC on melanoma cell death *in vivo* and the potential modifying effect of topical CpG, tumors of mice treated with DTIC were compared with tumors treated with combination therapy and phosphate-buffered saline (PBS) control. Apoptotic cells within the tumor mass were identified by TUNEL staining, and the frequencies of these cells were compared among the groups (Figure 3a and b). DTIC therapy significantly increased the fraction of apoptotic cells within the tumor mass (11 ± 3 vs 1.6 ± 0.2%), as did combination therapy with DTIC and topical CpG (7.8 ± 1.7%). There was little difference between tumors treated with DTIC alone and DTIC/CpG. TLR9 agonists are potent stimulators of DC activation. To determine if local DC cell phenotype modulation occurs at the treated tumor site, the DC cell populations within the tumors were phenotyped by flow cytometry (Figure 3c and d). Tumors treated with DTIC and topical CpG demonstrated the appearance of a distinct $B220^+CD8^+DEC205^-CD11c^+$ population. This population was induced by topical CpG application, as it appeared in tumors treated with topical CpG alone (data not shown). Thus, DTIC induces apoptotic tumor cell death that is not modified within 48 hours by CpG, and epifocal CpG application promotes $B220^+CD8^+$ DC accumulation within the tumor. We also asked whether topical CpG application would promote the infiltration of other cellular subsets. The generation of IFN-producing killer DC (IKDC) has been described after the exposure of natural killer (NK) cells to CpG *in vitro* (Chan *et al.*, 2006). We find that the fraction of $CD11c^+$ cells within treated tumors that is $NK1.1^+$ $GR\text{-}1^-$, corresponding phenotypically to this population is increased roughly two- to threefold (Figure 3c and d). This subpopulation is distinct from the $B220^+CD8^+$ DC subset described above as determined by differential CD8 staining (data not shown).

Both CD4 and CD8 cells participate in the tumor response to DTIC/CpG therapy

Optimal tumor immunotherapy requires both $CD4^+$ T cell- and $CD8^+$ T-cell-mediated responses (Schuler-Thurner *et al.*, 2002). To determine which immune cells participate in response to DTIC/CpG therapy, we determined the effect of antibody-mediated depletion of $CD4^+$ cells, $CD8^+$ cells, and NK receptor-bearing cells upon tumor growth. Groups of mice were inoculated with tumors and treated with anti-CD4, anti-CD8, or anti-NK antibodies immediately before the DTIC/CpG treatment and weekly thereafter. Analysis of tumor growth (Figure 4a) demonstrated that $CD4^+$ cell depletion abrogated any inhibition of tumor growth due to DTIC/topical CpG therapy. An obvious effect of $CD8^+$ cell depletion was also noted and confirmed in a survival analysis (Figure 4b) ($P<0.002$ compared with non-depleted). Unlike melanoma therapy models in which peritumoral CpG is administered (Sfondrini *et al.*, 2004), we noted only a very modest inhibition of protective effect after NK cell depletion using the NK1.1 antibody.

DISCUSSION

As the molecular and cellular mechanisms of immunologic tumor rejection have become better understood, specific stimulation of the host's immune system to induce melanoma tumor rejection has been attempted. Approaches to tumor-specific immunotherapy of melanoma have included chemoimmunotherapy combining IFN-α or IL-2 with DTIC therapy, use of tumor lysates and whole tumor vaccines, tumor-derived peptides, DC-based vaccination protocols, and adoptive transfer of tumor-specific T cells (Kadison and Morton, 2003). These approaches have so far met with little

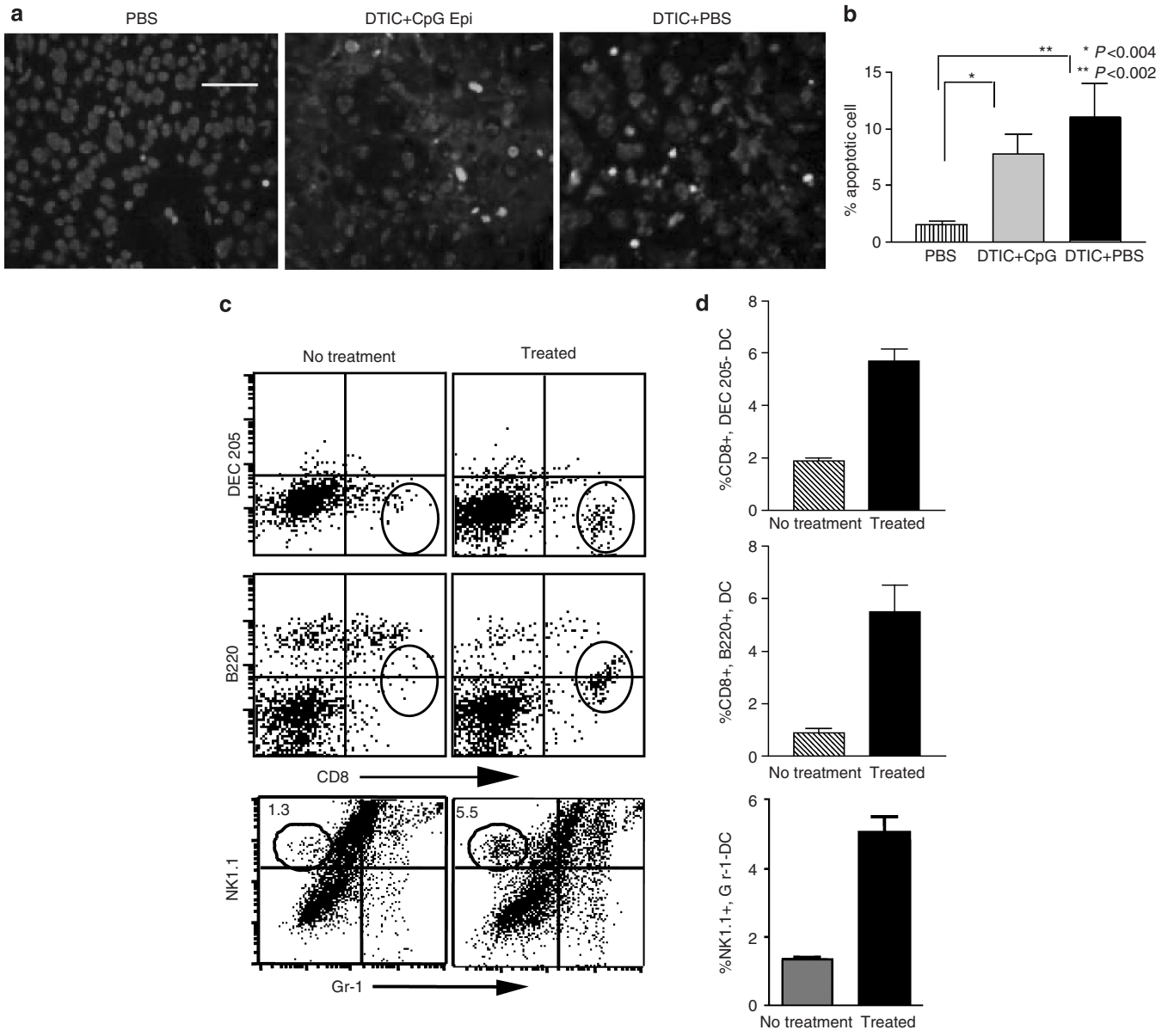

Figure 3. DTIC induces apoptosis in melanoma tumor-bearing mice and CpG recruits B220$^+$CD8$^+$ dendritic cells. B16 tumor bearing mice (four per group) were treated with DTIC followed by either CpG topically overlying the tumor mass (DTIC + CpG Epi) or saline (DTIC + PBS). Tumors were collected for immunohistochemical analysis 1 day after treatment. (**a**) Apoptotic cells were detected using TUNEL assay on tissue sections and cells were enumerated using DAPI nuclear staining as demonstrated (original magnification × 400, scale bar = 35 μm). (**b**) Percentage of apoptotic cells were then determined. Data presented are means ± SEM from 10 optical fields per histological section from three mice per group. (**c**) After four treatment cycles, tumors (three per group) were removed for flow cytometric analysis. Live cells were gated for CD11c$^+$ cells and plotted for DEC 205 and CD8α (**c**, upper panel) and were also stained for pDC (plasmacytoid DC) markers (CD8^{high}B220^{int}) (**c**, middle panel) and NK cell markers (NK1.1 and Gr-1) (**c**, lowest panel). (**d**) The bar graphs show the average percentage and SEM of CD8^{high}DEC205^{low}, CD8^{high}B220^{int} cells, and NK1.1$^+$ Gr-1$^-$ cells among CD11c$^+$ cells recovered from tumors in three separate experiments. *$P < 0.004$, **$P < 0.002$.

success in the clinic or they are complex and labor-intensive approaches that limit the potential for widespread use.

DTIC induces melanoma cell death by apoptosis. However, melanoma cells, owing in part to the biological function of their parent melanocytes, display an intrinsic resistance to apoptosis (Soengas and Lowe, 2003). Thus, the incidence of apoptotic melanoma cells is at most 10% as noted here (Figure 3). This may explain the lack of efficacy of this agent when used alone (Figure 1). We found no evidence for an increase in short-term DTIC cytotoxicity toward melanoma after combination therapy (Figure 3). We reasoned that the modest DTIC-induced cell death would nevertheless be sufficient to load DC with tumor antigen *in situ* and that the local activation of DC would enhance tumor-specific immune responses, leading to more effective therapy. Our results (Figure 1) confirm that the combination of DTIC and

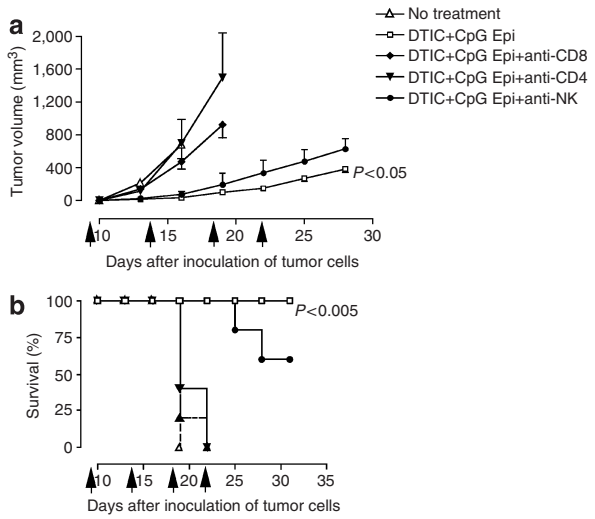

Figure 4. CD4$^+$ and CD8$^+$ cells play an active role in tumor regression after chemoimmunotherapy using topical or intratumoral CpG. Tumor-bearing mice were treated with anti-NK, anti-CD8, or anti-CD4 antibody 1 day before starting chemoimmunotherapy with DTIC and topical CpG. Antibody treatment was repeated weekly to ensure the persistent depletion of the relevant cell population. Mice without DTIC and CpG served as an additional control. Groups of mice treated with anti-CD8 or anti-CD4 antibody showed no inhibition of tumor growth (**a**) and lower survival ($P<0.002$ for CD8 and $P<0.002$ for CD4 compared to DTIC/CpG) (**b**). Elimination of NK cells had no significant effect on the therapeutic response to DTIC/CpG treatment.

local TLR9 stimulation using topical CpG improves the therapeutic response over either agent used alone.

The potency of TLR9 agonists such as CpG in promoting CTL responses has led to their experimental use to treat cancers. Human trials have shown modest responses, and treatment has been associated with systemic side effects (Krieg, 2007). Topical CpG administration results in the cross-priming of subcutaneous antigen and in the rapid activation of DC within the skin-draining lymph nodes (Najar and Dutz, 2007). We demonstrate here that the topical CpG application after DTIC treatment of subcutaneous melanomas results in the generation of CTL to tumor antigen (Figure 2) and in the accumulation of B220$^+$CD8$^+$ DC within the tumors (Figure 3). The generation of CTL to a previously defined B16-dependent tumor antigen is consistent with *in situ* DC loading and activation. The B220$^+$CD205$^-$CD8$^+$ phenotype of accumulating DC (CD11c$^+$ cells) within the tumor is identical to the phenotype of TLR9-activated type 1 IFN-producing plasmacytoid DC (O'Keeffe *et al.*, 2002). Such activated plasmacytoid DC secrete high local levels of IFN-α, have direct CD4 T-cell-activating function, and induce cross-presentation of tumor antigen for the activation of CD8 T cells by conventional DC (Liu, 2005). We have also identified the accumulation of cells bearing both DC markers (CD11c) and NK cell markers (NK1.1) within treated tumors. Such cells have been shown to possess direct cytotoxic activity against murine melanomas (Taieb *et al.*, 2006). Cell depletion studies confirm that CD4$^+$ and CD8$^+$ cells (T cells and/or DC), but not the NK1.1$^+$ cells, critically contribute to the therapeutic response (Figure 4). This further suggests that systemic immunity has been obtained. However, the efficacy of this treatment against systemic (pulmonary) metastases, not directly treated by the CpG adjuvant, remains to be determined.

Standard approaches to chemoimmunotherapy for melanoma have used the systemic administration of both chemotherapeutic and immunotherapeutic agents with a consequent increase in toxic effects without a difference in survival rate (Sasse *et al.*, 2007). In the clinical setting of cutaneous melanoma metastases, the multiplicity of tumors may make them un-resectable or not amenable to isolated limb perfusion therapy and new therapeutic approaches are needed. Topical TLR agonist application to promote an immune response, combined with agents to promote tumor cell destruction, may provide a novel approach. The topical TLR7/8 agonist imiquimod induces type 1 IFN responses and promotes plasmacytoid DC accumulation within murine melanoma tumors (Palamara *et al.*, 2004). Topical application of imiquimod similarly enhances the immune response to single melanoma lesions destroyed by cryosurgery in mice (Redondo *et al.*, 2007). Topical imiquimod has recently been used in attempt to control cutaneous metastatic melanoma in humans with mixed results (Green *et al.*, 2007; Shistik *et al.*, 2007).

Parenteral use of TLR9 agonists in advanced human melanoma may be associated with partial responses (Pashenkov *et al.*, 2006). The systemic use of TLR9 agonists together with local tumor destruction using cryotherapy results in increased immune responses and survival in a murine melanoma model (den Brok *et al.*, 2006). Our results highlight the potential therapeutic value of topical CpG application for the management of cutaneous metastases of malignant melanoma and provide an alternative to the use of topical imiquimod. In clinical situations where multiple cutaneous metastases are present, limited tumor cell destruction by DTIC may be more tolerable than tumor cell destruction by cryotherapy. As topical CpG in combination with DTIC induced systemic CTL activity against melanoma cells, the possibility exists that this treatment may induce immune responses to widespread metastatic disease. The relative clinical efficacy of topical TLR7 and TLR9 agonists in the treatment of cutaneous or widespread metastases by promoting *in situ* DC loading and activation and enhancing the effects of either chemotherapy or cryotherapy for the treatment of melanoma remains to be determined. Given the apparent safety and efficacy of topical CpG in the treatment of cutaneous metastatic melanoma in mice, preliminary clinical trials of this therapy in humans should be encouraged.

MATERIALS AND METHODS

Animals

C57Bl/6 mice (6- to 12-week old) were obtained from Charles River Laboratories (Wilmington, MA) and housed in a specific pathogen-free environment. Animal experiments were approved by the Animal Care Committee of the University of British Columbia.

Cell lines and reagents

EL4 thymoma and B16-F10 murine melanoma cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in complete medium consisting of RMPI 1640 medium (Invitrogen, Burlington, ON) supplemented with penicillin G (100 U ml^{-1}), streptomycin sulfate (100 μg ml^{-1}), 5×10^{-5} M β-mercaptoethanol and 10% fetal bovine serum. DTIC was purchased from Bayer Health Care (Toronto, Canada).

Synthesis of peptides and oligonucleotide

Purified, single-stranded, phosphothioated ODN-1826 (5′-TCCAT-GACGTTCCTGACGTT-3′) containing CpG motifs was synthesized and dissolved in 50 μl PBS and DMSO (1:1) and stored at −20°C until use. ODNs were prepared and purified to >80% purity at the Nucleic Acid and Peptide Synthesis Facility of the University of British Columbia. TRP2 peptide (SVYDFFVWL: corresponding to amino acids 180–188 of tyrosinase-related protein 2; Bloom *et al.*, 1997) was synthesized at the same facility.

Subcutaneous tumors

Tumors were induced by subcutaneous injection of 1×10^5 melanoma cells in 25 μl PBS on the flank of mice. Treatment was started 9 days thereafter, when tumors reached an average volume of 4 mm^3. One treatment cycle consisted of an intraperitoneal injection of DTIC (80 mg kg^{-1}) followed at 24 hours by the topical (epifocal) application of CpG-ODN (250 μg in 100 μl PBS) over the subcutaneous tumors after tape stripping as described (Najar and Dutz, 2007) or by intratumoral injection of CpG-ODN (50 μg in 25 μl). Cycles were repeated every 4th day. The tumor volume was scored every 3 days as half the product of the length × width^2. In survival studies, animals where censored when the tumor measured 1,000 mm^3.

Flow cytometry

DCs were prepared from tumors removed after four treatment cycles by digesting the tumor with 1 mg ml^{-1} collagenase D (Roche, Basel, Switzerland) supplemented with 40 μg ml^{-1} DNase (Boehringer Mannheim, Mannheim, Germany) in complete medium at 37 °C for 60 minutes. The released cells were collected in complete medium with 0.1 M EDTA buffer. DCs were enriched using Ficol (NycoPrep, Oslo, Norway). Cells were incubated on ice with anti-FcR mAb (2.4G2; American Type Culture Collection (ATCC), Rockville, MD) to block Fc-binding sites. DCs were stained on ice for 30 minutes with labeled antibodies before FACS analysis on a FACSCalibur (BD Biosciences San Jose, CA) using CELLQuest software. Antibodies to CD8α (clone 53-6.7), CD45R/B220 (clone RA3-6B2), CD11c (clone HL3), NK1.1 (clone PK136), and Gr-1 (clone RB6-8C5) were purchased from Pharmingen (San Diego, CA). Anti-DEC 205 (clone NLDC-145) was from Cedarlane (Hornby, Canada).

In vivo depletion of effector cells

Mice ($n = 5$ per group) with palpable tumors were randomly divided into five groups. Mice were treated with 400 μg relevant isotype antibody (rat IgG1 antibody), anti-NK1.1 (clone PK136; ATCC, Rockville, MD), anti-CD4 (clone GK1.5), or anti-CD8 (clone 53-6.72) depleting mAbs given intraperitoneally on day 9 and repeated weekly. Cell depletion was verified by flow cytometry of peripheral blood. Antibody treatment resulted in >95% depletion of the targeted cell population as detected using separate antibodies. Mice were treated with DTIC and ODN-CpG starting on day 10, and tumor volumes were scored every 3 days. A control group was left untreated.

Assessment of *in vitro* cytotoxic activity

Mice were euthanized 6 days after the last treatment cycle or as required by tumor growth. A standard 4-hour ^{51}Cr release assay was performed after *in vitro* stimulation of splenocytes as described (Najar and Dutz, 2007). Single-cell suspensions of splenocytes (2×10^7 per well) were stimulated *in vitro* with irradiated (15,000 rad) B16 cells (2×10^6 per well) in six-well culture plates in complete medium supplemented with 50 IU ml^{-1} recombinant human IL-2 (Pharmingen). After 5 days culture, effector cells were mixed with ^{51}Cr-labeled target cells. The target cells B16 and EL-4 cells were pre-pulsed, or not, with 10 μg ml^{-1} TRP2 peptide for 90 minutes.

Immunohistochemical staining and TUNEL assay

Tumor samples were formalin-fixed and paraffin-embedded. Fluorescence-based TUNEL staining of tissue sections was performed using a kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions. Sections were counterstained with DAPI (Vector Laboratories, Burlingame, CA). Images were captured with a Zeiss Axioplan fluorescence microscope equipped with a COHO-CCD camera. Apoptotic cells were counted in 10 fields (selected stepwise) per section at ×400 using one section per animal.

Statistical analysis

Groups of two data sets were compared using two-tailed Student's *t*-tests. Tumor growth curves were compared using Tukey's honestly significant difference (HSD) test in a *post hoc* analysis. Survival curves were compared using a Kaplan–Mayer log rank analysis. Results were displayed using Prism 3 (GraphPad, San Diego, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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